NARCOSIS AND CELL DIVISION IN COLPODA STEINII

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Introduction

For many years it has been known that a great number of chemical and physical agents can modify the course of mitosis and induce the development of abnormal division figures. Among the many experimental agents that have been employed to upset normal cell division are the following: basic dyestuffs (Politzer, 1924), narcotics (Politzer, 1931; Nemec, 1904; van Regemorter, 1926; Shaklevich, 1938; Ludford, 1936; Geiersbach, 1939), ether (Häcker, 1900; Schiller, 1909; Rosenfeld, 1932), alcohol (Krantz, 1938), X-radiation (Alberti and Politzer, 1923; Pfuhl and Küntz, 1939), radium (Whitman, 1933), ultraviolet radiation (Stevens, 1909), high and low temperatures (Bury, 1913; Bleier, 1930; Vintemberger, 1930; Kemp and Juul, 1931), and hypotonic solutions (Lewis, 1933). The instructive point to be gathered from a survey of such studies is that, irrespective of the agent employed, the induced morphological changes in cell division are quite similar. Thus, within certain limits of concentration or intensity of the experimental agency, disorientation of the chromosomes in the division figure, delay in polar movement and scattering of the chromosomes, "amitosis," pyknosis, dissolution of the achromatic figure, inhibition of cytokinesis, and formation of bi- and multinucleate cells are common effects.

The relative lack of specificity in results produced by such diverse experimental treatment makes the formulation of any complete explanation for these phenomena difficult. However, the suggestion of Ludford (1936) to the effect that metabolic changes may have a bearing on the production of anomalous divisional behavior becomes significant in light of recent observations indicating that special portions of the cell's metabolism are directly associated with certain physiological activity states (For details see Bodine (1934); Robbie, Boell and Bodine (1938); Deutsch and Raper (1938); Goddard and Smith (1938); Horowitz (1940); Pease (1941); Allen and Goddard (1938); van Schouwenberg (1938); Clowes and Krahl (1939); MacLeod (1941); Ormsbee (1941), and Fisher and Stern (1942)). From these studies it becomes apparent that a definite relationship exists between a portion of the over-all metabolism and cell or tissue activity. The use of narcotics as respiratory poisons and the deductions made by Fisher and his associates from data obtained by this type of treatment are of considerable significance in connection with this problem. The present study is an attempt to relate changes in cellular metabolism during division with the appearance of abnormal mitoses. The results of this study are in essential agreement with those of Fisher et al in that a respiratory parallelism can be demonstrated in *Colpoda steinii* by use of ethyl carbamate or chloral hydrate. The "activity system" of Fisher (that portion of the overall

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respiration that is most sensitive to narcotic inhibition) appears to be somehow associated with the maintenance of normal mitosis, for when it is differentially suppressed by appropriate narcotic concentrations, abnormal nuclear figures appear. These aberrant mitoses are believed comparable to those described in the literature that have been induced by many different agents. Barring such experimental intervention, the nuclear complex of *Colpoda steinii* is remarkably stable, unlike certain other members of the family Colpodidae (Burt, Kidder and Claff, 1941). Because of this stability it was selected for the study to be described.

I am indebted to Professor George W. Kidder, Arnold Biological Laboratory, Brown University, for his stimulating interest during the course of this investigation.

Materials and Methods

Colpoda steinii was employed as experimental material throughout this investigation (for species designation see Burt, 1940). Although this ciliate cannot be readily grown in the absence of bacteria (food organisms) it offers several distinct advantages over other forms, notably, its rapid growth to high concentrations and the clarity of the mitotic changes during division (Burt, Kidder and Claff, 1941).

The ciliates were grown in sterile distilled water seeded with the coliform bacterium *Aerobacter cloacae*. One liter Erlenmeyer flasks were used as culture vessels. The procedure employed in culturing was as follows: 400 mls. of distilled water were placed in the flasks which were then plugged and autoclaved at 15 lbs. pressure for 20 minutes. Twenty-four hour Kolle flask cultures of *A. cloacae* on agar were used as food. The bacterial growth of two such Kolle flasks was harvested in 30 mls. of sterile distilled water, and 10 mls. of the resulting suspension were added aseptically by means of a pipette to each of two Erlenmeyer flasks containing the sterile distilled water. In this manner fairly uniform suspensions of food organisms were obtained. Stock cultures of *C. steinii* were carried in tubes with Aerobacter as food. Each flask was inoculated with one ml. of these tube cultures containing 12 hour cultures of ciliates (in the logarithmic growth phase). The Erlenmeyer culture flasks were then incubated 24–30 hours at room temperature before the ciliates were sacrificed. By adhering to this procedure an abundance of logarithmic actively dividing cells could be consistently obtained.

All culture vessels, pipettes and miscellaneous glassware employed in culturing the ciliates were autoclaved 20 minutes at 15 lbs. pressure to prevent extraneous bacterial contamination inasmuch as optimum growth of *Colpoda steinii* obtains with Aerobacter alone as the food organism (Kidder and Stuart, 1939). Throughout all cultural procedure rigid bacteriological technique was followed to obviate the difficulties arising from contamination of the cultures.

In the cytological studies the cells were removed from the culture flasks and centrifuged at slow speed for four minutes in 50 ml. centrifuge tubes. The supernatant was then removed by means of an aspirator and more organisms added to the tubes from the culture. Following a second centrifugation, giving adequate numbers of organisms, the ciliates were placed in 250 ml. Erlenmeyer flasks containing 50–75 ml. of freshly bacterized solution of narcotic whose effect was to be tested. Equal amounts of bacterial suspensions were added to each experimental and control solution in making up the dilution of the reagent employed. When

the desired time of treatment had elapsed (one hour) the cells were again packed, the supernatant removed, and the cells placed on coverslips by means of capillary pipettes. It was found convenient and satisfactory to employ 25 per cent acetic acid in absolute alcohol for fixation. The nuclear details were found well preserved after fixation for five minutes in this reagent. The Feulgen technique was used exclusively in making the cytological preparations. This was necessitated by the fact that differentiation of the nuclear details is difficult with haematoxylin or other stains because of the retention of the dye by the division cyst walls. Best staining results were obtained by hydrolyzing in N/1 HCl at 60° C. for 12 minutes and staining in the fuchsin sulfurous acid for six hours. The stained preparations are then washed three times for four-five minutes each in HCl-Na-Bisulfite solution, placed for ½ hour in running tap water, passed through the lower alcohols to 95 per cent and stained with fast green, dehydrated in absolute alcohol, cleared in xylol and mounted in damar.

It was customary to run these cytological experiments in four sections as follows: by centrifuging in four tubes the contents of the mass culture flasks were roughly quartered. The organisms obtained by centrifugation from three of these tubes were employed experimentally and accordingly treated with various concentrations of reagent. The cells from the fourth tube were always set up with bacterized distilled water and thus served as controls for the treated organisms. The control ciliates were always mechanically treated in a manner identical with that received by the experimental organisms. By this method aberrations in the mitotic or divisional processes could always be attributed safely to the effect of the inhibitor used.

For the respiration studies two 24–30 hour cultures set up as described were centrifuged and the cells so concentrated were resuspended in bacterized M/200 phosphate buffer at pH 7.0. One and one half mls. of this cell-buffer suspension was added to the Warburg vessels. Following equilibration (15 minutes) four readings were taken at ten minute intervals to determine the normal uninhibited respiration. The graded concentrations of inhibitor to be tested (made up in M/200 phosphate buffer at pH 7) were then dumped from the side bulbs and following a second equilibration period of ten minutes readings were again taken at ten minute intervals for one hour to determine the extent of respiratory inhibition. Temperature was controlled at 25° C. ± 0.1° C.

It was considered advisable to set up three vessels with M/200 phosphate buffer in the side bulbs to serve as controls for the experiments. In addition to these normal controls two more vessels were run with 1.5 ml. suspension of Aerobacter equivalent to the bacterial suspension employed in the experimental vessels. The final computed values for oxygen consumed per hour could therefore be corrected for not only an increase or decrease in normal control respiration but also for the negligible amount of oxygen consumed by the food organisms both before and after inhibition. The fact that organisms of the genus Colpoda encyst when the supply of food bacteria is depleted (Taylor and Strickland, 1938; Kidder and Stuart, 1939) necessitated the addition of bacteria to the experimental suspensions. Because of the presence of the bacteria, absolute values of oxygen consumed by the ciliates would be very difficult if not impossible to ascertain. However, where only relative rates of respiration are sought, as in this instance, the food organisms introduce no serious technical difficulty.

A simple technique was employed as a means of testing the degree of growth suppression in different concentrations of inhibitor. Although this method lacks the refinement of a cell counting technique, the results are believed to be roughly comparable. In these experiments four tubes were set up for every narcotic solution to be tested. The final volume of the narcotic solution following bacterization was 3 mls. In addition to this series of four tubes for every narcotic concentration tested through the range under investigation, four more tubes were set up with bacterized distilled water to serve as controls. Following inoculation with one loopful of logarithmic Colpoda, the growth in each of the four inhibited series and controls was read at 12, 24, and 36 hours. Control growth was arbitrarily designated as four plus, and growth in the experimental tubes designated as three plus, two plus, one plus, plus-minus, and minus depending on the degree of inhibition. The range of inhibitor concentration where growth was completely suppressed was termed the zone of complete growth inhibition. In these experiments aseptic procedure was not adhered to in either experimental or control tubes as over the period of time these determinations were made it was felt that extraneous bacteria would not significantly modify the results.

In assaying the effectiveness of the narcotic concentration tested in disrupting mitosis only cells in which the polar migration of daughter micronuclei was obviously retarded were counted as affected (compare Fig. 1-*H* with Fig. 1-*E*). As will presently be pointed out in detail, *Colpoda steinii* usually divides twice within a division cyst wall to produce four daughter ciliates. The aberrant nuclear divisions were accordingly expressed as per cent of all cells counted in mitosis between the first metaphase and the telophase of the second division. This method offered a reliable means of obtaining a quantitative estimate of the damage induced by the agents employed. It is to be emphasized, however, that the delay in polar migration of the daughter micronuclei was not the only observable defect resulting from narcosis. It merely served as a convenient method of evaluating the extent of mitotic derangement. The other changes concomitant with the polar defect will be considered elsewhere.

Potassium cyanide, potassium ferricyanide, sodium arsenite, iodoacetate, various carbamates and chloral hydrate were tested at varying concentrations for their effect on the division mechanism. Ethyl carbamate and chloral hydrate were most thoroughly studied in this respect and were also selected as inhibitors for the respiration studies.

Observations

Normal division

The organisms of the genus Colpoda normally reproduce within division cysts (for details see Kidder and Claff, 1938; Burt, Kidder and Claff, 1941). The trophic ciliates (Fig. 1-*A*) round up prior to division, dedifferentiate and secrete the cyst wall within which the ensuing fissions occur, *C. steinii* usually dividing twice to form four daughter cells. These stages are depicted in Figure 1, *A–G*. The first changes in the nuclear complex marking the onset of division are to be found in the macronucleus. This organelle loses its trophic ellipsoidal shape, becomes rounded, and the polar chromatin aggregates break up into irregular masses attaining the general configuration shown in Figure 1-*B*. Meanwhile the

micronucleus proceeds through the prophasic changes first marked by swelling then condensation of chromatin to form striae from which the chromosomes are formed. These micronuclear transformations culminate in the metaphase configuration shown in Figure 1-*B* with the chromosomes oriented parallel to the long axis of the spindle. At this stage the macronuclear chromatin may be considered as divided into two portions, one consisting of the irregular masses centrally disposed; the other, being peripherally located and in optical section, seems to be plastered to the macronuclear membrane giving rise to a beaded appearance. The anaphase is depicted in Figure 1-*C*. At the termination of metaphase, the division figure appears to break into two parts followed by the rapid movement of the daughter halves to polar positions at opposite sides of the macronucleus (Fig. 1-*D* to *E*). Upon displacement of the daughter micronuclei by 180° the macronucleus elongates and constricts centrally. Cytokinesis soon follows at the completion of which the daughter micronuclei immediately pass into prophase and the same series of events is repeated with the result that four daughter cells are produced as shown in Figures 1-*F* and 1-*G*. Motor and oral organelles are differentiated in the four daughters and swimming movements are taken up within the cyst wall until finally the cyst membrane is ruptured and the ciliates escape into the surrounding medium. No visible nuclear extrusion has been observed during divisional phases in this species (Burt, Kidder and Claff, 1941).

Experimental modification of mitosis

Striking differences were observed in the effectiveness of the various inhibitors employed in disrupting the divisional mechanism. Ethyl carbamate was found to be quite active in this respect, however, and because of various other advantages offered by this compound, notably its high solubility and effectiveness over a relatively wide concentration range, it was most extensively studied. Potassium cyanide, potassium ferricyanide, sodium arsenite (neutralized solution), and iodoacetate were without effect up to toxic concentrations. Related carbamates produced changes similar to those induced by ethyl carbamate and the results of treatment with chloral hydrate are believed to be entirely comparable to those caused by inhibition with the urethanes. In the following account the cytological changes induced by these substances will be considered.

Ethyl carbamate: Striking changes in the course of mitosis were obtained in concentrations of ethyl urethane ranging from 0.5 to 2.0 per cent. The treatment did not appear to affect a particular stage of mitosis, however, but inhibition of any divisional stage in progress at the time of exposure seemed to obtain. A similar observation was made by Ludford (1936) for this narcotic. In view of

FIGURE 1. *Colpoda steinii* ×1400. *A*, trophic organism; *B–G*, normal division phases; *H–L*, aberrant divisions produced by 1.3 per cent ethyl carbamate. *B*, Micronucleus in full metaphase of first division. *C*, Micronucleus in anaphase. *D*, micronucleus moving to poles. *E*, Micronucleus continuing polar movement; macronucleus elongating. *F*, Micronucleus at metaphase of second division. *G*, Second division completed. Nuclei of daughter cells returning to trophic condition. *H*, Failure of micronuclear migration; macronuclear chromatin aggregrates more diffuse; reappearance of Binnenkörper. *I*, Similar to *H*; arrested polar movement of micronuclei; macronuclear chromatin diffuse; Binnenkörper material reappearing. *J*, Segregation of both daughter micronuclei into one cell following first division. *K*, Arrestment of division at micronuclear metaphase. *L*, Both micronuclei at one pole of elongated macronucleus.

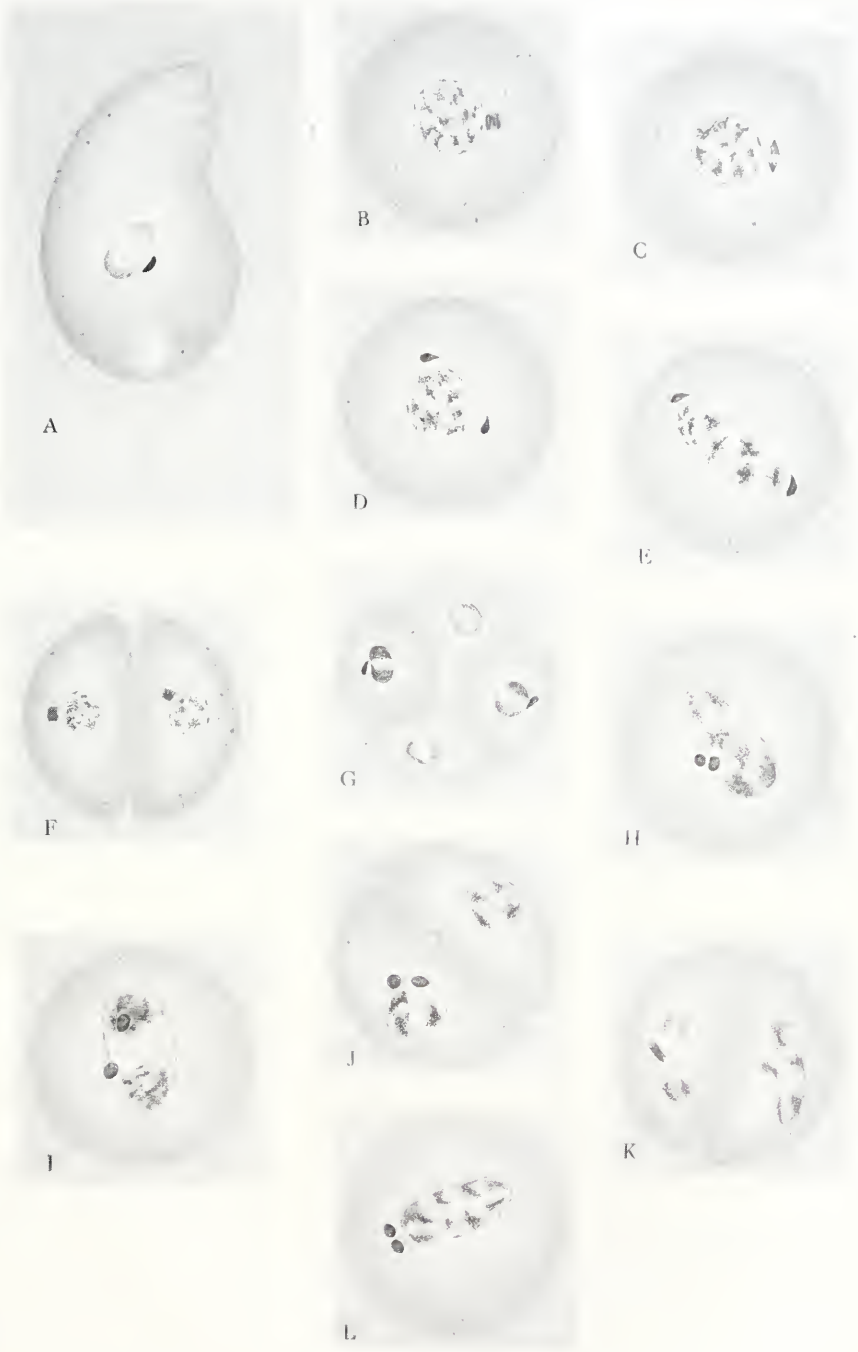

FIGURE 1

this fact, it is not at all surprising that a great variety of aberrant divisional types were observed. Because of this almost infinite variation in the nuclear features associated with narcosis, it is exceedingly difficult to evaluate the activity of the narcotic on any other basis than its effect on polar movement of daughter micronuclei.

With ethyl urethane this polar defect is exhibited through a concentration range of 0.5 to 2.0 per cent. Thousands of cells were counted following treatment with various narcotic concentrations (for complete data on this and the following observations see Burt, 1942). In Figure 2 these data are plotted semilogarithmically. The defective polar movement increases from 0 at 0.5 per cent urethane to a maximum of around 21 per cent at 1.5 per cent urethane concentration. This maximum value is probably determined by the number of ciliates in the first or second divisions whose micronuclei are between metaphase and telophase. During these stages one aspect of inhibition of cell division is expressed by lag in poleward movement.

As pointed out, however, the effect of urethane in appropriate concentrations on dividing *C. steinii* is characterized not only by delay of polar movement, but also by changes in the state of aggregation of both macro- and micronuclear chromatin and also by abnormal configurations of the dividing nuclear complexes. Some of these changes are illustrated in Figures 1-*H* to 1-*L*.

In Figure 1-*H* the polar defect is very well shown. At this phase of division, judging by the elongation of the macronucleus, the daughter micronuclei should have attained polar positions in the cell as is shown in Figure 1-*E*, a normal division figure. On the contrary, the daughters are centrally located near the middle of the macronucleus. A somewhat similar situation obtains in Figure 1-*I*, although the appearance is by no means as striking. In Figure 1-*L* another possible orientation of the nuclear complex is shown. Here the daughter micronuclei are terminally located at the same pole. One result of such aberrant behavior on the part of the daughter micronuclei is illustrated in Figure 1-*J*. In this instance cytokinesis and division of the macronucleus has occurred, but the micronuclei, due to failure in assuming polar positions, have both been segregated into one daughter cell. In Figure 1-*K* inhibition of division has occurred at the metaphase.

In all of these cases the aggregation of the macronuclear chromatin has been changed to some extent. In general, most of the macronuclear chromatin in the treated cells exhibits a tendency to become plastered to the nuclear membrane rather than maintain a central disposition characteristic of the normal dividing nucleus. Concurrently, the aggregates become more diffuse and varying degrees of fusion are shown (Fig. 1-*I*).

A noteworthy feature of the urethane-treated cells is the behavior of the Binnenkörper or plasmasomal material. Normally this substance, which stains lightly with fast green in tropic ciliates, is not in evidence in preparations immediately after the onset of nuclear division. However, in narcotized cells it reappears and is found dispersed around the diffuse chromatin aggregates of the macronucleus.

The morphological behavior of the chromatin and plasmasomal material in narcotized cells is suggestive of a partial reversion to a trophic nuclear condition. It is possible that under the influence of the narcotic and consequent suppression of normal nuclear division this tendency, although normally expressed at the termination of cell division, becomes dominant. At any rate, the appearance of the inhibited cells in no way suggests an *immediate* inhibition of all nuclear activity.

It is more likely that certain changes continue and give rise to the modified division figures observed.

Indicative of the fact that any phase of cell division is susceptible to the effects of narcosis is the diversity of aberrant division figures produced as a result of treatment. A continuous series of aberrant figures representing all stages of division may be constructed.

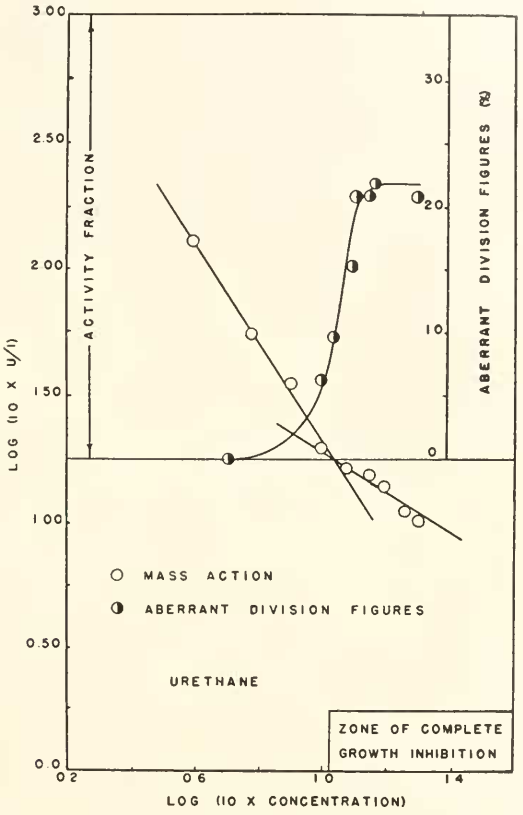

FIGURE 2. Summary of complete urethane data. Log $(10 \times U/I)$, aberrant division figures (per cent) and zone of complete growth inhibition plotted against Log $(10 \times$ concentration of urethane).

Other carbamates: The affects of n-propyl, phenyl, n-amyl, iso-amyl, and ethyl-n-methyl carbamate on cell division were also studied. None of these substances were tested through as wide a range of concentration in small graded increments as was ethyl carbamate. However, it was found that these closely related compounds produced divisional changes qualitatively identical with those of ethyl urethane. The ability to inhibit micronuclear migration is common to all members of this group of substances. Similarly, the associated changes in arrangement and aggregation of macronuclear chromatin and the bizarre division figures described for ethyl urethane were also induced.

Chloral hydrate: The activity of this compound in disturbing the normal progress of division was assayed in graded concentrations of 0.1 per cent increments between 0.05 and 0.5 per cent. The morphological changes induced were similar to those described for the urethanes, but the percentage of chloralized cells in which the polar defect appeared was never high. Moreover, the concentration range for effective induction of the polar defect is quite narrow (0.1–0.3 per cent). The maximum number of deranged mitoses was only 3.9 per cent, a small value compared with the maximum ethyl urethane value of more than 20 per cent.

In excess of 0.3 per cent chloral hydrate the delay in poleward movement and the changes in the nuclei characteristic of cells narcotized in the lower concentrations rather abruptly disappear. In these higher concentrations retrogressive changes are induced which are expressed as condensation, diffuse staining and chromatolysis of the macronucleus and pyknosis of the micronucleus. The incidence of these changes, although slight to moderate at 0.4 per cent, increases rapidly with still higher concentrations until in 0.5–0.6 per cent chloral hydrate all of the organisms, both trophic and those in division, are frankly moribund. These degenerative changes are physiologically and morphologically reversible in some cells at least after one hour exposure at 0.6 per cent. If the cells are washed free of narcotic, packed by centrifugation and resuspended in freshly bacterized distilled water, growth will ensue. It is impossible to determine, however, whether or not every cell so treated is viable.

In summary, chloral hydrate affects cell division in a manner quite similar to ethyl carbamate and related compounds. However, the effective range of concentration for the production of these changes is relatively narrow, and at no concentration are the numbers of deranged divisions high. A possible explanation for this difference in activity will be outlined.

Effect of other inhibitors: The following compounds in the concentrations indicated were tested for their effect on the divisional mechanism:

Inhibitor	Concentrations
Potassium cyanide	0.005M to 0.000,05M
Potassium ferricyanide	0.050M to 0.005M
Sodium arsenite	0.001M to 0.000,25M
Monoiodoacetic acid	0.005M to 0.000,5M

These metabolic poisons throughout the concentrations designated were uniformly ineffective in the production of aberrant divisions of the type described for the carbamates and chloral hydrate. When cells were exposed to the lower concentrations of these inhibitors no change could be detected cytologically in the progress of division. However, at higher concentrations retrogressive changes characterized by extreme condensation and pyknosis of both macronuclei and micronuclei occurred. These changes were comparable to those induced at high concentrations of chloral hydrate and are considered as definite signs of cell damage as opposed to the milder type of change occurring with moderate narcotic concentrations.

Growth inhibition

As a correlative procedure in connection with the cytological and metabolic studies, the effect of ethyl urethane and chloral hydrate on suppression of growth in cultures of *C. steinii* was tested.

In Figure 3 are tabulated the results from one typical determination with ethyl urethane. Fairly close correspondence in the degree of inhibition among the four experimental series was obtained. It is apparent from these results that growth is only slightly affected by 0.5 per cent urethane (3 plus to 4 plus growth). However, with increasing concentrations of narcotic, suppression of growth becomes more pronounced until at 1.0 per cent urethane no cell division at all can be detected. Similarly, Ormsbee (1941) found that 1.0 per cent urethane would completely inhibit the growth of populations of the ciliate *Tetrahymena geleii*.

CHLORAL HYD.		EXP. 1			EXP. 2			EXP. 3			EXP. 4		
SERIES	PERCENT CONC.	HOURS			HOURS			HOURS			HOURS		
		12	24	36	12	24	36	12	24	36	12	24	36
A	.025	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
B	.05	++++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++
C	.1	+	±	±	±	+	±	±	±	±	±	±	±
D	.2	–	–	–	–	–	–	–	–	–	–	–	–
E	.3	–	–	–	–	–	–	–	–	–	–	–	–
F	.4	–	–	–	–	–	–	–	–	–	–	–	–
G	CONTROL	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

FIGURE 3. Inhibition of growth in cultures of *Colpoda steinii* with various concentrations of ethyl carbamate. Four plus growth represents growth in untreated controls.

URETHANE		EXP. 1			EXP. 2			EXP. 3			EXP. 4		
SERIES	PERCENT CONC.	HOURS			HOURS			HOURS			HOURS		
		12	24	36	12	24	36	12	24	36	12	24	36
A	0.5	+++	++++	++++	++++	++++	++++	+++	++++	++++	++++	++++	++++
B	0.8	+	+	+	+	++	++	+	++	++	+	+++	+++
C	0.9	±	+	+	±	±	+	±	±	+	+	+	+
D	1.0	±	±	±	±	±	±	±	±	±	±	±	±
E	1.1	–	–	–	–	–	–	–	–	–	–	–	–
F	1.2	–	–	–	–	–	–	–	–	–	–	–	–
G	CONTROL	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

FIGURE 4. Inhibition of growth in cultures of *Colpoda steinii* with various concentrations of chloral hydrate. Four plus represents growth in untreated controls.

In Figure 4 are represented the results of an analogous determination made with chloral hydrate. Here complete inhibition of growth takes place in 0.2 per cent of the narcotic.

Comparison of the two sets of experimental results is instructive in indicating the differences in effective concentration ranges of the two inhibitors. In the case of ethyl carbamate an increment of 0.6 per cent in concentration must be made from the point where its effect is first noted until complete inhibition occurs (from 0.5 to 1.1 per cent). With chloral hydrate this increment is only 0.15 per cent or one fourth that of urethane. This is correlated with the brief range of effectiveness of chloral hydrate in the induction of aberrant division figures.

Respiratory metabolism

As pointed out in a preceding section, overall oxygen consumption in a cell or tissue does not necessarily have uniform significance as regards a specific function. Perhaps the most convincing evidence for this is the interpretation of nacrotic inhibition data made by Fisher and his colleagues (for review see Fisher, 1942).

Equations expressing the relationship between enzyme and inhibitor may be derived from the Law of Mass Action (Warburg, 1927; Warburg and Negelein, 1928; Fisher and Öhnell, 1940). As required by the Mass Action formulation, when the logarithm of the concentration of inhibitor (narcotic) is plotted against the logarithm of $\frac{\text{Uninhibited respiration}}{\text{Inhibited respiration}}$ a straight line should result. Non-linearity of mass action curves so constructed is believed to indicate the presence of at least two parallel respiratory systems in Arbacia eggs and in yeast (Fisher, 1941a, b; Fisher and Henry, 1940; Fisher and Stern, 1942).

The respiration of *Colpoda steinii* was tested in graded concentrations of urethane and chloral hydrate with the object of obtaining evidence for such respiratory discontinuity in this organism. The results of respiratory inhibition as measured by the Warburg technique between concentrations of 0.4 and 2.0 per cent urethane are plotted in Figure 2. At a concentration of urethane between 1.0 and 1.2 per cent a break in the mass action curve occurs at which point respiration is inhibited by 35 per cent. This is taken as evidence for the existence of two separate respiratory mechanisms that differ in sensitivity to narcotic inhibiton.

In Figure 2 is also shown the relationship between this respiratory data, growth inhibition, and aberrant nuclear behavior at various levels of narcotic inhibition. The increase in pathological cell divisions is related to the degree of suppression of the upper limb of the mass action plot representing the inhibition of the more highly narcotic-sensitive fraction of metabolism. It is also interesting to note the correspondence between the concentration of urethane giving complete growth inhibition (1.1 per cent) and the concentration at which the break in the mass action curve occurs (approximately 1.1 per cent).

A discrepancy exists in these relations, however, for the maximum number of aberrant nuclear divisions is not obtained until a concentration of 1.4 to 1.5 per cent urethane is reached. This may be due to experimental error in part, but the theoretically more attractive possibility is that an overlapping in the inhibition of the two respiratory systems occurs. In accordance with this view the resting system would begin to be inhibited before the activity system was completely suppressed. This point will be dealt with more fully in a following section. Apparently, however, following complete inhibition of the more narcotic sensitive fraction (activity system of Fisher) not only is growth of the culture suppressed, but the incidence of abnormal divisional figures attains a maximum.

In Figure 5 is summarized the effect on respiration, growth and the derangement of cell division brought about by graded concentrations of chloral hydrate between 0.0125 and 0.6 per cent. These results resemble those obtained with ethyl urethane. Non-linearity of the mass action curve is characteristic. Also a fair correspondence exists between the concentration giving complete growth inhibition (0.2 per cent) and the concentration at which discontinuity of the respiratory inhibition becomes apparent (0.1 per cent). Unlike urethane, however, the per-

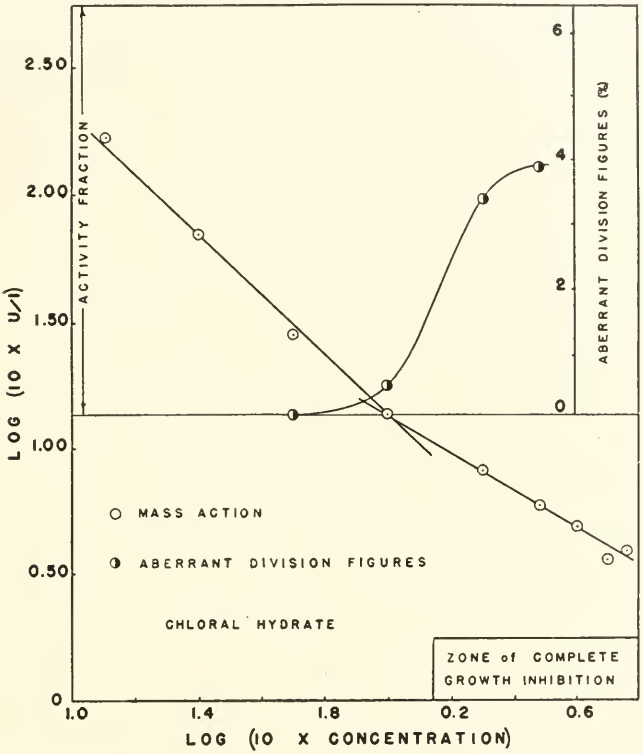

FIGURE 5. Summary of complete chloral hydrate data. Log $(10 \times U/I)$, aberrant division figures (per cent) and zone of complete growth inhibition plotted against Log $(10 \times$ concentration of chloral hydrate).

centage of aberrant nuclear divisions induced by chloral hydrate is never high, the maximum being in the vicinity of 4 per cent. This maximum is not obtained until the cells are exposed to 0.3 per cent chloral hydrate, or 0.2 per cent in excess of the concentration at which discontinuity in the respiratory data is expressed. Again, a possible explanation for this may be an overlapping in the inhibition of the activity and resting systems whereupon after a certain concentration is exceeded, both are inhibited simultaneously. That the more sensitive fraction indicated by chloral hydrate represents about 45 per cent of the total respiration (taking the break in the curve as the end point) in contrast to 35 per cent with urethane makes this possibility plausible. The higher value of respiratory inhibition down to the

point where discontinuity is displayed in the mass action curve therefore represents the activity system plus the added respiratory decrease caused by the suppression of part of the less sensitive fraction.

Discussion

It is believed that under the experimental conditions adhered to in this study the data indicate the presence of at least two fractions of metabolism in *Colpoda steinii*. These differ in their sensitivity to inhibition by either chloral hydrate or urethane. One portion of this parallel respiratory mechanism, the activity fraction, appears to be associated with the maintenance of normal cell division. In general, this is in agreement with the observations of Fisher and his colleagues (1940–1942). This conception is based on the fact that discontinuity in the relation between concentration of narcotic and its effect in depressing respiration of these ciliates is expressed by mass action treatment of the data. The factors that might invalidate such an interpretation have been pointed out by Fisher and Stern (1942), but it is believed that these factors were not operative in this investigation.

Complete and quantitative separation of activity and resting fractions of the total metabolism by differential narcotic inhibition is considered here as extremely unlikely. Four findings in this report support the view that parallel inhibition of the two systems occurs in an intermediate range of narcotic concentrations:

1. The increase to a maximum in the incidence of nuclear aberrations after mass action discontinuity has been expressed. This is considered most significant and was found with both urethane and chloral hydrate (See Figs. 2 and 5).

2. The difference of 10 per cent between the activity respiration indicated by urethane (35 per cent) and that of chloral hydrate (45 per cent).

3. The delay in obtaining complete growth suppression with chloral hydrate after discontinuity in the mass action curve has been expressed.

4. The brief effective concentration range of chloral hydrate in inducing abnormal division figures and the low maximum for the polar defect obtained with this reagent.

Nuclear divisional activity persists at narcotic concentrations in excess of those at the break in the mass action curves. The maximum number of nuclear abnormalities with urethane does not occur until a concentration of 1.5 per cent of the narcotic is reached, and 0.3 per cent chloral hydrate is necessary to attain the corresponding maximum. Hence, the relation between the activity fraction, as determined by the respiratory inhibition curves, and divisional activity is not precise. It is believed that this failure of complete correspondence between the production of aberrant mitoses and discontinuity of the respiratory data is due to parallel or simultaneous inhibition of both activity and resting systems in an intermediate range of narcotic concentrations. In effect, this means that continued nuclear activity is possible even after the break in the mass action curves occurs because the activity system is not completely suppressed at this point. Mitosis therefore continues in an increasingly abnormal fashion until the activity metabolism is finally completely eliminated.

The conception of overlapping or parallel inhibition of both respiratory mechanisms also implies that the slopes of the two lines describing the reactions cannot

be accurately descriptive of the relation between the narcotics on the separate systems even though the existence of at least two respiratory mechanisms is apparent. Furthermore, the value of 35 per cent representing the activity respiration, as determined by urethane inhibition, is not an absolute value for the percentage of the total energy involved in cell multiplication. However, this figure corresponds fairly well with the 28 per cent found for *Tetrahymena geleii* by Ormsbee (1941) and the 30–40 per cent of the overall respiration associated with the maintenance of cell division in sea urchin eggs reported by Krahl and Clowes (1939).

The value of 45 per cent for activity respiration, determined by chloral hydrate, can be accounted for by overlapping in the inhibition of the two respiratory mechanisms. The additional 10 per cent activity respiration indicated by this drug would thus represent part of the resting respiratory mechanism that was involved at the narcotic concentration where the break in the mass action curve occurred. Failure to completely suppress growth at a concentration of 0.1 per cent chloral hydrate (at break in logarithmic plot) likewise is indicative of this inhibition overlap. With the increase in narcotic to 0.2 per cent cell multiplication ceases because of the more complete elimination of the activity system.

When the progress of mitosis is suppressed by appropriate narcotic concentrations nuclear changes described earlier in this report as reorganizational are induced. Higher concentrations of narcotic inhibit these trophic changes (as 3 per cent urethane or 0.5 per cent chloral hydrate). At these concentrations the nuclei become condensed and pyknotic. The morphology described as resulting from varying degrees of suppression of the activity system obtained at lower narcotic concentrations are not seen at these higher drug levels. Thus in the production of nuclear abnormalities of the type described, two mechanisms appear to operate:

1. The inhibition of the activity system with associated suppression of the progress of mitosis.

2. Reorganization of the nuclei toward the trophic condition at that stage in division where inhibition has occurred.

The trophic changes are considered here to be dependent upon the maintenance of the resting metabolism of the cell (narcotic resistant system). As more of this less sensitive respiratory system is inhibited with increasing narcotic dosage, a point is finally reached when reorganization toward the trophic morphology cannot occur. Concomitantly pyknosis sets in and the nuclear picture is completely altered. Where the resting fraction is inhibited simultaneously with the activity fraction a restriction on the effective production of aberrant divisional figures results. The brief range of effectiveness and low maximum for nuclear abnormalities obtained with chloral hydrate can be accounted for on this basis.

The conception of overlapping with respect to narcotic inhibition postulated in the foregoing account is supported by the observation of Fisher (1941a) that benzoate inhibits the resting system in sea urchin eggs before inhibiting the activity system. Here it is necessary to inhibit 50 per cent of the respiration before inhibition of cleavage occurs. The condition described for *C. steinii* in its behavior with narcotics appears to represent an intermediate situation between the theoretical, where precise quantitative separation of the two systems would occur, and the juxtaposition of the two systems with respect to sensitivity to benzoate described for sea urchin eggs.

It is to be emphasized that the narcotic concentration required to inhibit cell multiplication in cultures corresponds closely with that at the point in the mass action curves where discontinuity is expressed, i.e., when the activity system, as determined by this method, is inhibited growth of the culture ceases. To suppress nuclear activity, however, more complete elimination of the activity system appears to be necessary, hence the delay in attaining the maximum number of abnormal division figures.

The cytological effects of narcotics on dividing *Colpoda steinii* are considered here as unique, but are comparable to the results obtained with these agents on many other cell types. Furthermore, it is apparent that in addition to narcotics many chemical and physical agents induce quite similar changes in the course of cell division. In general, the interpretation placed on this non-specificity of effect is that regardless of the agent employed there is somehow brought about a modification of the intracellular colloid state. This common result of experimental treatment would account for the similarities of morphological variation (see Ludford, 1936; von Lehotzky, 1938; Kemp and Juul, 1931; M. R. Lewis, 1933, 1934; Rosenfeld, 1932). This point of view, in light of the paucity of information available that is pertinent to the actual mechanics of these defects, appears to be well taken. However, the complexity of the protoplasmic organization in its reactions to various treatments must not be overlooked.

In the present study it has been shown that with gradual suppression of that portion of the cells' energy yielding reactions designated as activity metabolism, cell multiplication is suppressed and abnormal division figures are induced. It is not surprising that the divisional mechanisms drawing on these energy sources should reflect such reductions in energy production by abnormal behavior. However, it is difficult to visualize the manner in which this metabolic inhibition becomes morphologically expressed.

Possibly by interfering with cellular oxidation (narcosis) physical changes in the protoplasm result (von Lehotzky, 1938); this would relate the abnormal division figures obtained by narcotics to those induced by temperature changes, ether, carbon dioxide, hypo- and hypertonic solutions. All of these agencies have been demonstrated to affect the viscosity of protoplasm (for review, see Chamber's discussion in Cowdry's *General Cytology*). Conceivably, such experimental interference might exert its effect by modifying normal cyclical viscosity changes occurring during cell division (Heilbrunn, 1917; 1920; 1921).

No doubt such physical changes are significant for the normal progress of cell division and bear consideration in dealing with experimentally induced changes in mitosis. That such purely physical aspects fail to describe the entire sequence of events encountered in the complex dynamics of cell division is obvious. It may be, however, that they are the links between the biochemical phases involving cellular oxidations and the well known cytological manifestations of activity. Integrating factors for such an association are at present lacking. With regard to the present study, it can only be said that a certain portion of the overall metabolism is probably associated with these mechanisms. To postulate more than this would be the sheerest speculation.

Briefly, the negative results obtained in the preliminary work with cyanide, ferricyanide, arsenite and iodoacetate may be considered. The reactions of any of these inhibitors in the intact cell, with the possible exception of cyanide, are

complex. At the present time data concerning their effect on the metabolism of *Colpoda steinii* are lacking. Apparently, however, with the limited information concerning the morphological expression of treatment by these agents, their effects differ considerably from those of the narcotics tested. To take one example, cyanide does not seem to visibly alter the division mechanism until the occurrence of retrogressive changes resembling those induced by the higher doses of narcotics. Tentatively it may be suggested that cyanide, by virtue of its reaction at the oxygen end of the cellular oxidation chain where a large percentage of respiration is mediated, simultaneously inhibits both activity and resting systems. When a certain percentage of the resting metabolism has been suppressed, the retrogressive alterations are induced.

Summary

1. Ethyl carbamate has been demonstrated to be effective in inducing aberrant nuclear behavior during division of the ciliate *Colpoda steinii*. This activity is shared by other carbamates and chloral hydrate. These effects are described in detail.

2. A quantitative means of assaying cytologically the activity of these substances is described. On the basis of defective polar movement of daughter micronuclei ethyl carbamate was found to be more active than chloral hydrate. A possible explanation for this observation is postulated.

3. The results of preliminary work involving treatment of dividing cells with potassium cyanide, potassium ferricyanide, arsenite, and iodoacetate are reported. These agents apparently do not share the activity evidenced by the carbamates or chloral hydrate.

4. By means of the Warburg technique the effect of urethane and chloral hydrate on respiration of *C. steinii* was studied.

5. Discontinuity in the quantitative action of these agents on respiration is interpreted to indicate the existence of at least two fractions of metabolism. One of these, the activity system, is believed to be associated with the process of cell division.

6. Complete separation of these two systems is not considered probable, however, and caution is urged in the interpretation of discontinuity in Mass Action treatment of this and similar data.

7. The significance of the association of the appearance of aberrant division figures, growth inhibition and discontinuity in the relationship of narcotic concentration to respiratory inhibition is pointed out. Discrepancies in these relationships are discussed.

8. The bearing of metabolic inhibition on the general problem of the pathology of mitosis is discussed.

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